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TITLE: Reduction of Radiation- or Chemotherapy-Induced Toxicity by Specific
Expression of Anti-Apoptotic Molecules in Normal Cells

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Introduction

The toxic effects of radiation and chemotherapy on normal tissues is a significant problem for patients with breast cancer because those effects worsen quality of life (QOL) and hinder the ability to tolerate these conventional therapies at effective levels. Our long-range goals are to reduce the toxicity associated with radiation therapy or chemotherapy and to improve the QOL of patients with breast cancer. One mechanism by which radiation and chemotherapy produce toxic effects is through inducing apoptosis of normal cells in normal tissues. We hypothesize that ectopic overexpression of anti-apoptotic molecules will inhibit the radiation- or chemotherapy-induced apoptosis of normal cells and thereby reduce the toxicity of these treatment modalities. We have shown that overexpression of the anti-apoptotic gene Bcl-2 can protect normal breast epithelial cells from apoptosis *in vitro*, but whether this effect will reduce the toxicity associated with radiation or chemotherapy in patients is unknown. Cells that contain wild-type (wt) p53 typically react to the genotoxic stress of radiation or chemotherapy by upregulating the expression of p53, which binds to specific DNA sequences and activates specific genes, some of which activate apoptosis. To prevent expression of anti-apoptotic genes by cancer cells, we exploited the fact that many types of cancer cells lack or have mutated forms of p53 and are developing a construct in which expression of anti-apoptotic genes is driven a minimal promoter under the control of the wt p53 binding sequence. This strategy is expected to limit the expression of anti-apoptotic genes to normal cells, thus reducing the risk that breast cancer cells become chemo- or radioresistant because of inappropriate overexpression of anti-apoptotic molecules. We expect that cells under genotoxic stress (by being exposed to chemotherapy or radiation) will express higher levels of anti-apoptotic molecules owing to upregulation of p53. Finally, we will use LPD cationic liposomes to create a novel, nonviral gene delivery system for systemic delivery of these anti-apoptotic molecules to normal organs such as lung, liver, kidney, and spleen. This proposal is innovative in that it seeks to prevent or reduce the toxic side effects of conventional therapies (i.e., radiation and chemotherapy) by inhibiting the fundamental biological process of "apoptosis" that they induce in a wt p53-specific manner. The results obtained may lead to the discovery of effective ways to protect normal tissues from radiation or chemotherapy without reducing the efficacy of those treatments.

The specific aims proposed to meet these goals are as follows.

Specific Aim 1. To induce p53-dependent inhibition of radiation- or chemotherapy-induced apoptosis by anti-apoptotic molecules.

Specific Aim 2. To develop a promoter specific for wild-type p53-expressing cells, using wild-type p53-DNA binding sites upstream from a minimal promoter.

Specific Aim 3. To determine the extent to which anti-apoptotic molecules under the control of a novel p53-specific promoter (PGn) complexed with LPD can be used, under optimal conditions, to reduce the toxicity of radiation therapy or chemotherapy.

The goal for year 1 of the original application was to complete Specific Aim 1. Progress toward that goal is described in the remainder of this report.

Body

Key Research Accomplishments

Specific Aim 1. To induce p53-dependent inhibition of radiation- or chemotherapy-induced apoptosis by anti-apoptotic molecules. Our original hypothesis was that upregulation of wt p53 by radiation or chemotherapy will result in overexpression of anti-apoptotic molecules under the control of a minimal promoter downstream of p53 binding sites, which will in turn inhibit stress-induced apoptosis. We addressed this hypothesis in the following 2 subaims.

- 1-1. *Determine whether overexpression of Bcl-2 in normal cells can inhibit apoptosis in vitro and improve survival of cells.* Preliminary studies described in the original application showed that Bcl-2 could inhibit doxorubicin- (Adriamycin-) induced cytotoxicity. However, the extent to which Bcl-2 could inhibit the cytotoxicity of other genotoxic agents such as Taxol and γ -radiation was not known. Thus we established stable Bcl-2-overexpressing transfectants of normal human breast epithelial cells (MCF10A) (data not shown), the breast cancer cell line MDA-MB-231 (data not shown), and normal mouse fibroblasts (NIH3T3) (Fig. 1).

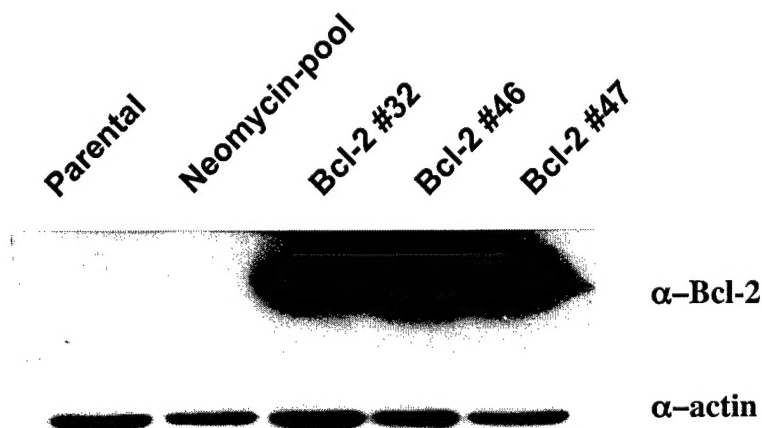


Fig. 1 Overexpression of Bcl-2 protein in NIH3T3 cells. Three clones (#32, #46, #47) showed higher Bcl-2 expression than the parental or neomycin pool by western blotting. Actin was used as a loading control.

Cytotoxicity in Bcl-2-overexpressing NIH3T3 stable transfectants was reduced compared with that in parental cells after treatment with paclitaxel (Taxol), doxorubicin (Adriamycin) or γ -radiation, as evaluated by the MTT assay (Fig. 2). Similar results were obtained with the MCF10A normal human breast epithelial cell line and the MDA-MB-231 human breast cancer cell line (data not shown). Moreover, neither MCF10A-Bcl-2 transfectants nor NIH3T3-Bcl-2 transfectants formed colonies in soft agar, which suggests that tumorigenicity is not increased by

the expression of Bcl-2. The reduction in cytotoxicity seems to be due to a reduction in apoptosis (as measured by fluorescence-activated cell sorting [FACS] analysis). We are repeating these experiments using TUNEL assays to confirm that the difference in cytotoxicity was due to apoptosis induced by the irradiation or the chemotherapy.

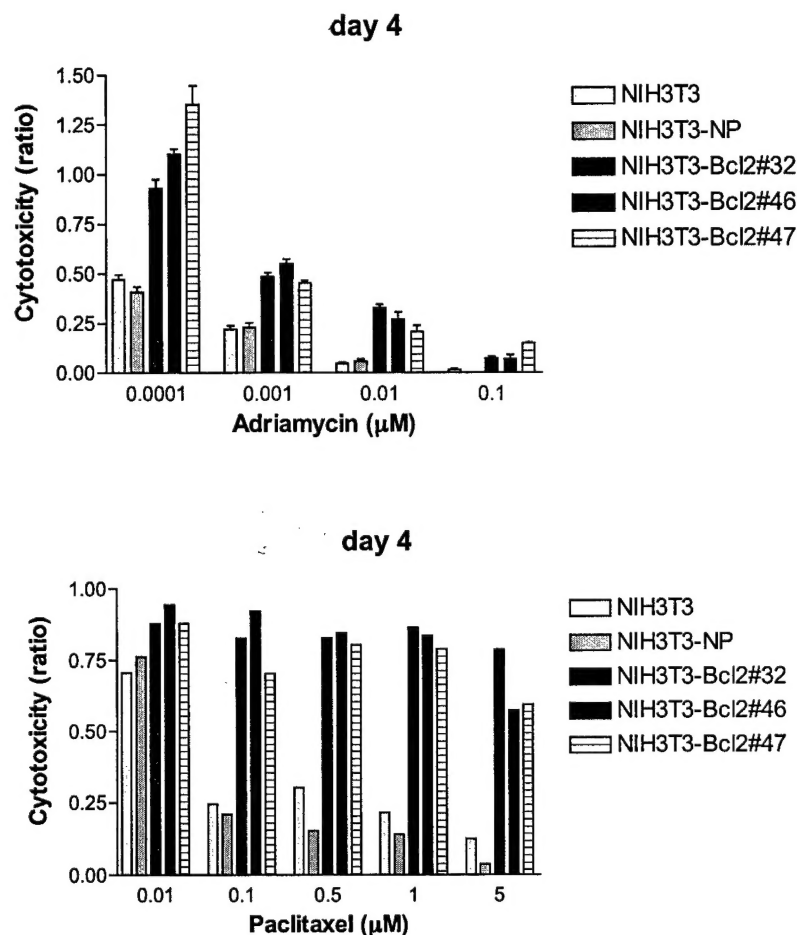


Fig. 2 Dose-response curves after exposure to doxorubicin or paclitaxel. Cell sensitivity to these agents was determined by MTT assay as follows. Cells were suspended in 96-well tissue culture plates and incubated at 37°C for 24 h. Cells were then treated with doxorubicin (adriamycin [A]) or paclitaxel (B) to obtain a dose-response curve. After incubation for 72 h, MTT solution was added to each well and the plates were incubated for another 4 h, after which 100 μ l of dimethylsulfoxide was added to solubilize the MTT-formazan product. Absorbance at 570 nm was measured with a microplate reader.

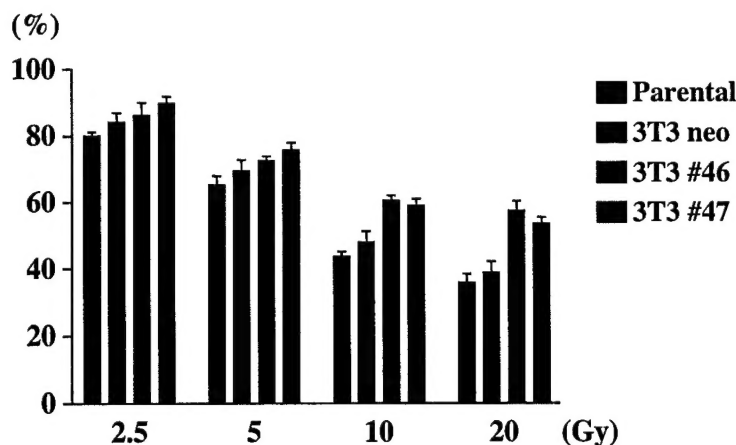
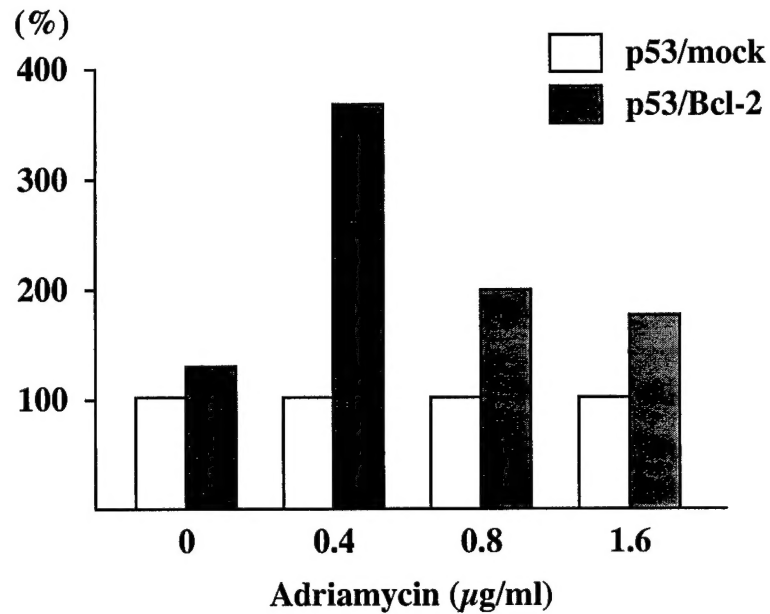


Fig. 3 Radioprotection by Bcl-2. Radiation-induced cytotoxicity was determined by MTT assay as follows. Cells were suspended in 96-well tissue culture plates and incubated for 24 h. Cells were then irradiated with doses of 2.5 to 20 Gy and incubated for another 72 h.

- 1-2. *Determine whether Bcl-2 overexpression in normal cells occurs only when those cells are stressed by irradiation or chemotherapy.* After confirming that Bcl-2 expression did inhibit cytotoxicity, we cloned the Bcl-2 in front of a polyoma early promoter located downstream of 13 copies of p53 binding promoter sequence (PG₁₃). As a control, Bcl-2 was cloned in front of a polyoma early promoter located downstream of 15 copies of a promoter sequence that does not bind p53 (MG₁₅). To test whether wild-type p53 induced by chemotherapeutic drugs could activate the PG13PyBcl-2 vectors, we tested A549 cells, which have the wild-type p53 gene. An increase in cell survival rate was noted in PG13PyBcl-2-transfected cells compared with that of the PG13Py-transfected cells, especially when treated with 0.4 µg/ml doxorubicin (Fig. 4a). We next examined the expression of p53 and Bcl-2 protein in A549 cells that had been transfected with PG13PyBcl-2 or PG13Py (Fig. 4b). In the PG13PyBcl-2-transfected cells, expression of p53 increased at 12 h after doxorubicin treatment and expression of Bcl-2 increased at 24–72 hours after treatment, indicating that PG13PyBcl-2-transfected cells could still express Bcl-2 proteins after treatment with doxorubicin and still showed reduced doxorubicin-induced cytotoxicity.

(A)



(B)

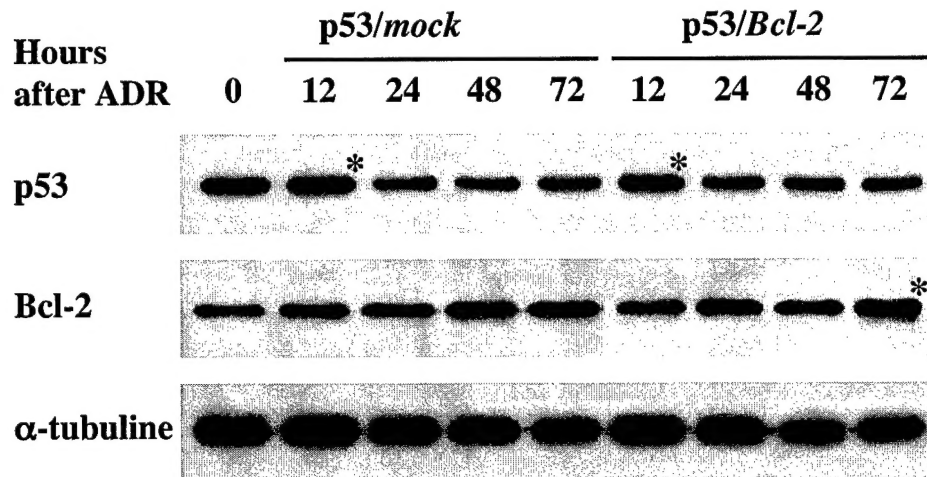


Fig. 4 Activation of p53 induced Bcl-2 expression and reduced the cytotoxicity of doxorubicin (Adriamycin). (A) To assess cell survival rate, PG13PyBcl-2 and luciferase vector were cotransfected into A549 cells and incubated for 24 h. Cells were then treated with doxorubicin at 0.4 to 1.6 μ/ml to assess their chemosensitivity. After incubation for 48 h, the activity of luciferase was measured. The luciferase activity of PG13Py-transfected cells was defined as 100%. (B) Expression of p53 and Bcl-2 were determined by western blotting. A549 cells were transfected with PG13PyBcl-2 or with PG13Py vector and treated with 0.4 μg/ml doxorubicin. Tubulin was used as a loading control.

In summary, Bcl-2 expression was upregulated when the PG₁₃ constructs were transfected into cells carrying wt p53, but not when transfected into cells carrying mutated or deleted p53. We also observed upregulation of Bcl-2 in cells exposed to the chemotherapy agent doxorubicin by the induction of p53.

Specific Aim 2 (To develop a promoter specific for wt p53-expressing cells, using multiple wt p53-DNA binding sites upstream from a minimal promoter) and Specific Aim 3 (Reduction of radiation- or chemotherapy-induced toxic effects in normal cells in mice bearing mutated p53 breast cancer xenografts) will be addressed in the second and third years of funding.

Reportable Outcomes

The findings described here were submitted for presentation at the upcoming DOD Army Breast Cancer meeting in September 2002. Two expression vectors of Bcl-2, one under the control of the polyoma early promoter located downstream of 13 copies of the p53 binding promoter sequence (PG₁₃) and the other under the control of the polyoma early promoter located downstream of 15 copies of p53 mutated binding promoter sequence (PG₁₃) were constructed. Stable transfectant cell lines that overexpress Bcl-2 were established for MDA-MB-231 cells (a p53-mutated breast cancer cell line), NIH3T3 cells (a wild-type p53 murine fibroblast cell line), and MCF10A cells (a wild-type p53 normal breast epithelial cell line).

Conclusions

We have shown that ectopic overexpression of Bcl-2 counters the cytotoxicity of doxorubicin (Adriamycin), paclitaxel (Taxol), and radiation. We also showed that Bcl-2 expression can be induced specifically in cells with wild-type p53, in particular under genotoxic stress, when cells were transfected with a heterogeneous promoter of a wild-type p53-specific promoter combined with Bcl-2 cDNA. When MCF10A or NIH3T3 cells were exposed to chemotherapy agents, p53 upregulation in these cells resulted in specific expression of luciferase. These findings indicate that normal cells expressing wild-type p53, but not p53-mutated or p53-deleted breast cancer cells, can be protected from at least some of the effects of radiation or chemotherapy by inducing the wt p53-specific expression of Bcl-2.

References

N/A

Appendices

N/A